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REVIEW

Oxidative stress and bivalves: a proteomic approach

D Sheehan[1], B McDonagh[2]

[1]*Proteomics Research Group, Dept. Biochemistry, University College Cork, Lee Maltings, Prospect Row, Mardyke, Cork, Ireland*
[2]*Departamento de Bioquímica y Biología Molecular, University of Cordoba, Campus de Rabanales, Córdoba 14071, Spain*

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Abstract

Bivalves are of major importance in aquatic ecology, aquaculture, are widely used as sentinel species in environmental toxicology and show remarkable plasticity to molecular oxygen. Excess reactive oxygen species (ROS) arising from molecular oxygen can cause oxidative stress and this is also a consequence of exposure to many common environmental pollutants. Indices of oxidative stress have therefore found favor as biomarkers of exposure and effect in environmental toxicology. However, there is a growing body of literature on the use of discovery-led proteomics methods to detect oxidative stress in bivalves. This is because proteins absorb up to 70 % of ROS leading to complication of the proteome. This article explores the background to these developments and assesses the practice and future potential of proteomics in the study of oxidative stress in bivalves.

Key words: bivalve; oxidative stress; mussel; clam; ecotoxicology

Introduction

Molecular oxygen (O_2) first accumulated on Earth ~ 2.3 billion years ago due to the appearance of photosynthesis. The redox characteristics of the Earth's atmosphere fundamentally altered from reducing to strongly oxidizing and living cells, with reducing internal environments, for the first time needed to expend considerable energy to survive the surrounding oxidizing environment. Oxygen is paradoxical in that it is on the one hand essential for the most efficient form of energy metabolism; aerobic metabolism. On the other hand, it is a potential chemical threat because it can lead to formation of reactive oxygen species (ROS) (Halliwell and Gutteridge, 2007; Winterbourn, 2008). These include species such as H_2O_2, the hydroxyl and superoxide radicals (Fig. 1) which are naturally formed in living cells especially in subcellular organelles such as the mitochondrion and endoplasmic reticulum. Cells developed elaborate strategies to cope with ROS as part of adaptation to their changed redox situation including antioxidant enzyme activities (e.g., catalases), small antioxidant molecules (e.g., glutathione, GSH; vitamin E) and redoxins (e.g., thioredoxins). Under normal circumstances, these defenses cope well with the levels of ROS produced and cells exist in a state of redox homeostasis. However, in certain circumstances, the balancing act between ROS production and antioxidant defences tilts in favor of build-up of ROS in the cell leading to a state of oxidative stress (Halliwell and Gutteridge, 2007).

Bivalves (informally including mussels, clams, oysters and scallops) comprise animals of the class Bivalvia in the phylum Mollusca which first appeared late in the Cambrian explosion (~ 530 million years ago) and eventually came to dominate over brachiopods in the Palaezoic era (Gould and Calloway, 1980). They are characterized by a shell which is divided from front to back into left and right valves, have adapted their gills as filter-feeding organs and often have a well-developed byssus apparatus allowing them to attach to rocky substrates (Bayne, 1976). The precise reason for the evolutionary success of bivalves is a matter for conjecture (Gould and Calloway, 1980; Miller, 1998), but it is evident that they have shown considerable resilience and now occupy niches in a

Corresponding author:
David Sheehan
Proteomics Research Group,
Dept. Biochemistry,
University College Cork,
Lee Maltings, Prospect Row,
Mardyke, Cork, Ireland
E-mail: d.sheehan@ucc.ie

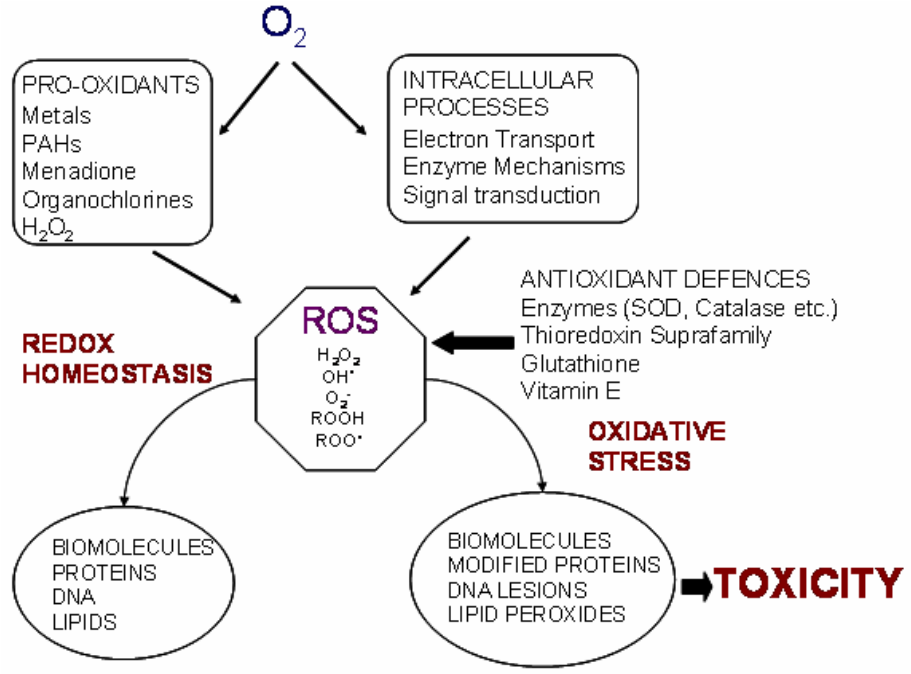

Fig. 1 Oxidative stress arises when there is an imbalance between production and neutralisation of ROS. These can arise from endogenous enzyme mechanisms or from exogenous sources such as metals and PAHs. Oxidative stress can result in modification of biomolecules, especially proteins and lead to toxicity.

wide range of aquatic environments. They are often the major macrofauna on rocky substrates of littoral, shallow sub-littoral and deep-sea vents (Bayne, 1976; Lutz and Kennish, 1993). Their filter-feeding habit adds greatly to their ecological significance in that bivalves are important calcium and carbon accumulators, they link primary producers (bacteria and phytoplankton) with higher organisms in aquatic food-chains and are responsible in tidal zones for filtration of the water body (Newell, 2004). Since most human interaction with the aquatic environment is concentrated along rivers, coastlines and estuaries (Halpern *et al.*, 2008), bivalves have importance in addition to their evolutionary and ecological significance. They are an important food-source for human populations and can be cultured for food and other reasons (Naylor *et al.*, 2000; Newell, 2004). Bivalves have genomes generally comparable in size to the human genome yet they are poorly represented in DNA sequence databases. For this reason, there is growing interest in bivalve genomics as a means of elucidating evolutionary, genetic and toxicological relationships (McKillen *et al.*, 2005; Tanguy *et al.*, 2008).

Because of their sedentary lifestyle, filter-feeding habit, abundance, ease of identification, tolerance to pollution and wide geographical distribution bivalves have found particular applications as sentinel species in environmental toxicology (ecotoxicology) (Bayne, 1979; Goldberg and Bertine, 2000). This has led to a considerable body of research focusing on using levels of particular biomolecules (biomarkers) to reveal effects of environmental pollutants and give insights to the pollution status of specific sampling sites (Cajaraville et al., 2000; Handy et al., 2003; Depledge and Galloway, 2005; Galloway, 2006). Since many environmental pollutants such as heavy metals, polyaromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and nanomaterials are known to be strongly pro-oxidant, much of this research has focused on biomarkers reflecting oxidative stress (Viarengo et al., 1991; López-Barea and Pueyo, 1998; Lesser, 2006; Valavanidis *et al.*, 2006). It has increasingly become clear that such studies also need to allow for effects due to non-pollution variables such as seasonality, nutritional status and the normal redox variations inherent in the bivalve lifestyle (Power and Sheehan, 1996; Manduzio *et al.*, 2004). More recently, interest has grown in extending this essentially hypothesis-driven biomarker approach to a discovery-driven approach in which high-throughput proteomics techniques are applied to bivalves to identify novel biomarkers for exposure to environmental pollution. This review explores how bivalves cope with ROS under normal circumstances, describes the biomarker approach to study of oxidative stress and reviews the technology and opportunities offered by proteomics in this important area of research. Bottlenecks to exploitation of this experimental approach are briefly described.

Reactive oxygen species and oxidative stress

In aerobic metabolism, molecular oxygen is eventually reduced to water. In this process, a

variety of oxygen derivatives, collectively called ROS, are naturally produced. These include the superoxide anion radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), peroxyl radicals (ROO•), nitrogen oxide (NO•) and hydroxyl radical (HO•) (Lesser, 2006; Winterbourne, 2008). Some ROS contain unpaired electrons (i.e. they are free radicals) whilst others are non-radical species (e.g., H_2O_2) (Fig.1). ROS can also be formed in response to a wide range of exogenous agents including radiation (X-ray, gamma, UV, or visible light in the presence of a sensitizer), metal ions, solvents, particulate matter, nitrogen oxides, ozone etc. (Davies, 2005). The HO• is the most important ROS in biology with an oxidation rate constant for protein components comparable to the rate of diffusion ~ 10^8-10 M^{-1} s^{-1} (Davies, 2005; Winterbourn, 2008). Due to its high reactivity HO• is quite non-specific in its targets for oxidation, whereas ROS with lower rate constants react more specifically (Davies, 2005; Winterbourn, 2008). ROS are generally removed rapidly by antioxidant mechanisms as they can affect major cellular components including lipids, proteins, carbohydrates and DNA and can ultimately lead to cell death (Fig. 1). ROS can cause serious toxicity because they are capable of interacting rapidly and efficiently with important biomolecular targets (Davies, 2005; Valavanidis *et al.*, 2006; Winterbourne, 2008). The most toxic ROS is HO• which can attack biological membranes in a diffusion-controlled fashion, initiating free radical-mediated chain reactions (Davies, 2005; Lesser, 2006; Winterbourn, 2008). ROS capable of diffusion across biological membranes (e.g., H_2O_2) can enter into numerous other reactions and so cause effects at a distance from their site of formation. The extent of damage which ROS can generate is dependent on a number of factors including: the concentration of target, the rate constant for reaction of oxidant with the target, the location of the target when compared to the site of oxidant formation, the occurrence of secondary events, the occurrence of oxidant-scavenging reactions and repair reactions (Davies, 2005; Winterbourn, 2008).

Oxidative stress in bivalves: what is "normal"?

Oxidative stress refers to a state where there is an imbalance between the generation and neutralisation of ROS by antioxidants, caused by excessive production of ROS, loss of antioxidant defenses or both (Halliwell and Gutteridge, 2007). Several aspects of bivalve biology make oxidative stress of especial significance. Bivalves can be exposed to relatively high levels of pro-oxidants as a consequence of their filter-feeding habit. Common environmental pollutants known to be pro-oxidants such as PCBs, PAHs, heavy metals and organochlorines are bioconcentrated within bivalves leading to oxidative stress (Viarengo *et al.*, 1991; Rodríguez-Ariza *et al.*, 1992, 1993, 2003; Cheung *et al.*, 2001; Downs *et al.*, 2002; Rodríguez-Ortega *et al.*, 2002; Valavanidis *et al.*, 2006). Moreover, intertidal animals exposed to air during the tidal cycle face particular challenges. These only survive dehydration because of the integrity of the seal between the two halves of their shell. However, this seal also cuts off access to oxygen from the animals' surroundings and oxygen inside the shell is rapidly depleted (Stachowitz *et al.*, 2007). Depending on local conditions, individual animals may be exposed to air for a comparatively short time-period (at the lower end of the intertidal zone) or for up to 7 h in every 12 (at the highest end of the intertidal zone). When exposed to air, animals soon experience hypoxia which can turn to anoxia in the more extreme circumstances of the highest part of the intertidal. This is a significant cause of mortality and individual animals can physiologically adapt to this challenge (Widdows *et al.*, 1979; Altieri, 2006; Stachowitz *et al.*, 2007). Normal aerobic metabolism is very quickly depressed with glycolytic fermentation progressively increasing (Widdows *et al.*, 1979; Widdows and Shick, 1985). On re-immersion, the bivalve opens its shell and experiences a rapid increase in oxygen level. Simultaneously, oxidative metabolism resumes (Widdows and Shick, 1985) resulting in a burst of ROS similar to the reperfusion-ischemia injury of mammals (Grace, 1994). Thus, as a routine part of their life-cycle, bivalves must have sufficient plasticity to cope with relatively large fluctuations in oxygen levels which can be further modulated by factors such as seasonality, pollution exposure and nutritional status (Viarengo *et al.*, 1991; Rodríguez-Ariza *et al.*, 1992, 1993, 2003; Power and Sheehan, 1996; Manduzio *et al.*, 2004; Lesser, 2006). Moreover, recent research (primarily in mammalian systems) suggests that, even in sub-stress scenarios, redox modifications may be an important aspect of normal cell signalling capable of triggering apoptosis (Biswas *et al.*, 2006; Ying *et al.*, 2007; Oktyabrsky and Smirnova, 2007; Poole and Nelson, 2008). While outside the scope of this review, bivalve species will no doubt be a fruitful area for future research into this important role of ROS.

Oxidative stress in bivalves: the biomarker approach

Bivalves have found especial favor as "sentinels" of environmental pollution and have come to be widely used in pollution surveillance programmes (Bayne, 1979; Goldberg and Bertine, 2000). Biomarkers are indices of the presence of chemical pollutants in particular environmental contexts (Cajaraville *et al.*, 2000; Handy *et al.*, 2003; Depledge and Galloway, 2005; Galloway, 2006). As has been pointed out (Power and Sheehan, 1996; Manduzio *et al.*, 2004) effects of environmental pollutants are to some extent dependent on biological (seasonal, nutritional and reproductive status) and environmental variables (e.g., temperature, exposure to sunlight). Bivalves have evolved robust biochemical and physiological defenses against chemical pollution and the varying levels of oxygen exposure to which they are subject (Widdows *et al.*, 1979; Widdows and Shick, 1985). They have an extensive battery of antioxidant defenses (Winston, 1991; Valavanidis *et al.*, 2006) and variations in these were found to be useful in environmental monitoring (Rodríguez-Ariza *et al.*, 1992, 1993, 2003; Sole *et al.*, 1996; Regoli *et al.*, 2002; Rodríguez-Ortega *et al.*, 2002; Manduzio *et*

al., 2004). Other important biomarkers include glutathione transferases (Fitzpatrick *et al.*, 1995; Lyons *et al.*, 2003), heat shock proteins (Sanders, 1993; Lyons *et al.*, 2003), DNA damage (Steinert, 1999), ubiquitination (Hofmann and Somero, 1995; Buckley *et al.*, 2001) , acetylcholine esterase, and metallothioneins (Vergani *et al.*, 2005; Lehtonen *et al.*, 2006). Investigation of these biomarkers was largely prompted by analogy with mammalian and/or *in vitro* toxicology and was therefore hypothesis-driven in that these biomarkers would be expected *a priori* to vary in response to chemical pollution. Notwithstanding this expectation, there have been some surprising instances where bivalve biomarkers show significant differences either in biochemical properties or toxicological response when compared to those from higher species (Livingstone, 1998; Vergani *et al.*, 2005; Sole and Livingstone, 2005). A significant body of literature has now grown up on biomarkers of oxidative stress in bivalves as pollution indices.

Proteomics approaches

The biomarker approach depends heavily on study of single variables which could either be serendipitously observed to occur in nature or predicted as likely effects of particular pollutants. Proteomics offers an alternative discovery-based approach for biomarker discovery of targets not necessarily predicted *a priori*.

Proteomic technologies

Each type of biological sample (organism, tissue, cell) expresses a characteristic subset of the proteins encoded in its genome. This protein population is further complicated by a range of possible post-translational modifications such as oxidation (Mann and Jensen, 2003; Spickett *et al.*, 2006; Sheehan, 2006). The proteome is defined as the complement of proteins expressed in a given biological system under a defined set of conditions. In contrast with the genome, the proteome is highly dynamic, changing with the type of cell and in response to variables such as nutritional or pollution status. Since chemical pollutants can alter the profile of proteins in the sample by altering protein structure (e.g., by post-translational modification; PTM) or by changing expression level of specific proteins (protein expression signature; PES) proteomics provides a potentially highly-sensitive means of detecting effects as well as offering potential insights to toxicity mechanisms (Heijne et al., 2005). This approach has the potential to reveal changes in the level/status of specific proteins against a background of unchanged proteins using high-throughput experimental platforms (reviewed in Sheehan, 2007) such as two dimensional electrophoresis (2DE) (Gőrg *et al.*, 2004) and mass spectrometry (MS) (Aebersold and Mann, 2003). While these approaches will be especially emphasized here, emerging proteomic technologies include protein microarrays (Cretich *et al.*, 2006) and the yeast two hybrid system (Zhu *et al.*, 2003). Sample origin and preparation are key variables in environmental proteomics since, as well as studying whole cell extracts prepared from plants or animals, selected sub-proteomes can be studied such as those prepared by affinity selection (Lee and Lee, 2004) and subcellular organelles (Kislinger *et al.*, 2006). Moreover, when working with genetically ill-defined animals such as bivalves (as opposed to inbred laboratory strains), it is necessary to have sufficient replicates to allow for inter-animal variation (biological replicates). In addition, multiple analyses are required to allow for experimental variation (analytical replicates). These are important facets of experimental design in environmental toxicology studies with bivalves (Karp *et al.*, 2005; Karp and Lilley, 2006; Monsinjon *et al.*, 2006).

2DE

2DE consists of sequential separation of proteins based first on isoelectric focusing (IEF) followed by orthogonal SDS PAGE (O'Farrell, 1975). Originally, a stable pH gradient was established in a polyacrylamide gel by including local buffering agents (ampholytes) when casting the gel. Proteins would migrate along the pH gradient until arriving at their isoelectric point (pI, the pH at which the protein has no net charge) and then stop moving. In this way proteins focus at their pI and the protein population separates in the rod gel into specific bands. In practice, it was technically demanding to achieve reproducible pH gradients in this system and there was a tendency for the entire gradient itself to migrate towards the cathode ("cathodic drift"; Lognonne, 1994). Immobilized pH gradients (IPGs) were later developed in which ampholytes were covalently immobilized along plastic strips (Görg *et al.*, 2000). After IEF, the focused strip is exposed to SDS PAGE sample buffer, laid across the top of an SDS PAGE gel and electrophoresed. When stained with coomassie or silver stain (Rabilloud, 1992), individual proteins become visible as spots which are usually sharply-defined (Fig. 2). Since few proteins have identical pI and relative molecular mass (Mr), especially high-resolution separations are achievable in which pairs of samples (e.g., test and control) can be compared. By increasing the size of the gel, up to thousands of spots may be evident but, even in the smallest gels, several hundred spots may be resolved. Separations are captured and analyzed by image analysis software (Corzett *et al.*, 2006). Independent labeling of lysines in test and control proteomes with up to three different fluorescent labels followed by their mixing and separation on a single gel allows comparison of tests and control samples without gel-to-gel variation, a technique called difference gel electrophoresis (DIGE) (Gade *et al.*, 2003). For the purposes of the present review, it should be noted that 2DE separations can also be interrogated to identify proteins targeted by specific redox lesions, thus revealing redox-related differences not detectable by PES alone (Sheehan, 2006).

Mass spectrometry (MS) methods

Chemical species with a given mass to charge ratio (*m/z*) describe a unique trajectory in an electromagnetic field *in vacuo* (Aebersold and Mann, 2003, Domon and Aebersold, 2006a). If the charge of all chemical species in a sample is identical, a mixture can be separated based on *m*

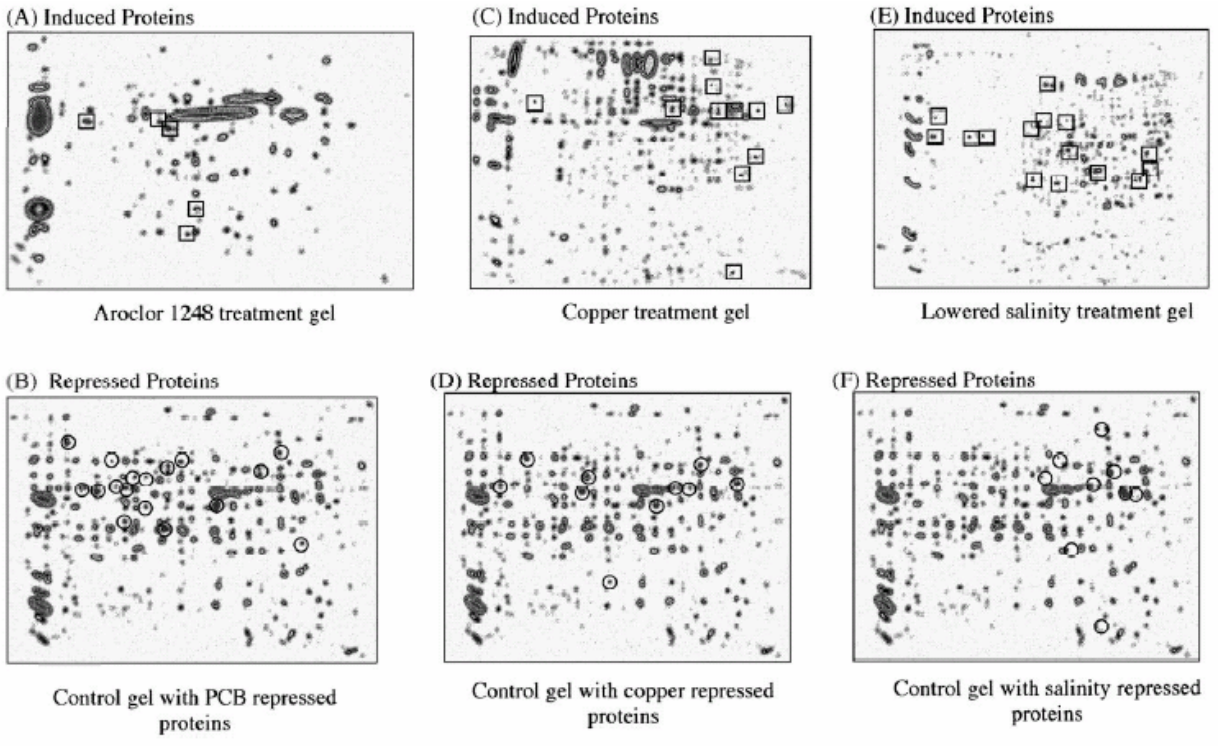

Fig. 2 Protein expression signatures (PES) of *Mytilus edulis*. Whole body proteomes separated by 2DE from animals exposed to (**A, B**) Arachlor 1248, (**C, D**) copper or (**E, F**) lowered salinity. These are composite gels (eight gels, four animals per treatment) and spots highlighted as being up-regulated (relative to controls) in at least 75 % of gels are highlighted with boxes (**A, C, E**). Spots down-regulated in 75 % of gels are highlighted as circles (**B, D, F**). From Shepard JL, Olsson B, Tedengren M, Bradely BP. Protein expression signatures identified in *Mytilus edulis* exposed to PCBs, copper and salinity stress. Mar. Environ. Res. 50: 337-340, 2000.

alone yielding a very accurate mass estimate of the order ± 0.1 %. This allows identification of proteins and peptides which are composed of well-understood structural components of known *m*. In proteomics, MS analyzes differences between proteomes at the level of individual proteins or is used to identify proteins from tryptic digests of spots in two dimensional gel separations. Protein identification is achieved either by peptide mass mapping (or fingerprinting) (Thiede *et al.*, 2003) or by peptide sequencing (Wu *et al.*, 2006). In both methods proteins are digested into a population of peptides with trypsin (or another protease) and these peptides are identified by comparison with predicted *m* values from sequence databases. Rapid interrogation/analysis of sequence databases is made possible with powerful bioinformatics programs (Wu *et al.*, 2006; Palagi *et al.*, 2006; Domon and Aebersold, 2006b).

Two common ionization methods are used for MS of proteins/peptides: Matrix-assisted laser desorption ionisation (MALDI) (Tanaka *et al.*, 1988) and electrospray ionization (ESI) (Fenn et al., 1990). MALDI is most commonly used in conjunction with a "time-of-flight" (TOF) detector so this platform is called MALDI-TOF (Fig. 3). Sample is mixed with a "matrix" of a material capable of absorbing some of the laser energy used to ionize the protein or peptide into the gas phase (e.g., sinapinnic acid) and then placed on a target for laser-induced ionization. Surface enhanced laser desorption ionization (SELDI) is a modification of MALDI increasingly used in biomarker discovery and ecotoxicology in which sub-proteomes are selected for categories of proteins on chips functionalized with selective chemical groups such as ion exchange (Merchant and Weinberger, 2000; Poon, 2007) (Fig. 4). ESI MS achieves ionization of intact proteins by progressively drying droplets containing sample (Fenn *et al.*, 1990). When the droplet becomes sufficiently small, the charges on ions now highly concentrated within the droplet repel each other so strongly that a coulombic explosion occurs ejecting structurally intact ions into the gas phase for separation/detection. This produces extremely accurate *m* determinations with a minimum of structural breakdown.

Tandem MS (also called MS/MS or MS^2) uses two MS sectors separated by a collision cell (Aebersold and Mann, 2003, 2006a; Wu *et al.*, 2006) (Fig. 5). A tryptic digest is first separated in one MS giving a unique *m* for each peptide. The peptide then passes through the collision cell where it fragments systematically giving a "ladder" of daughter ions which are separated on the second MS based on *m*. Since each fragment has a unique *m* and is composed of a limited number of chemical building blocks, the ladder can be read as a *de novo* amino acid sequence which can be used to screen sequence databases identifying the protein giving

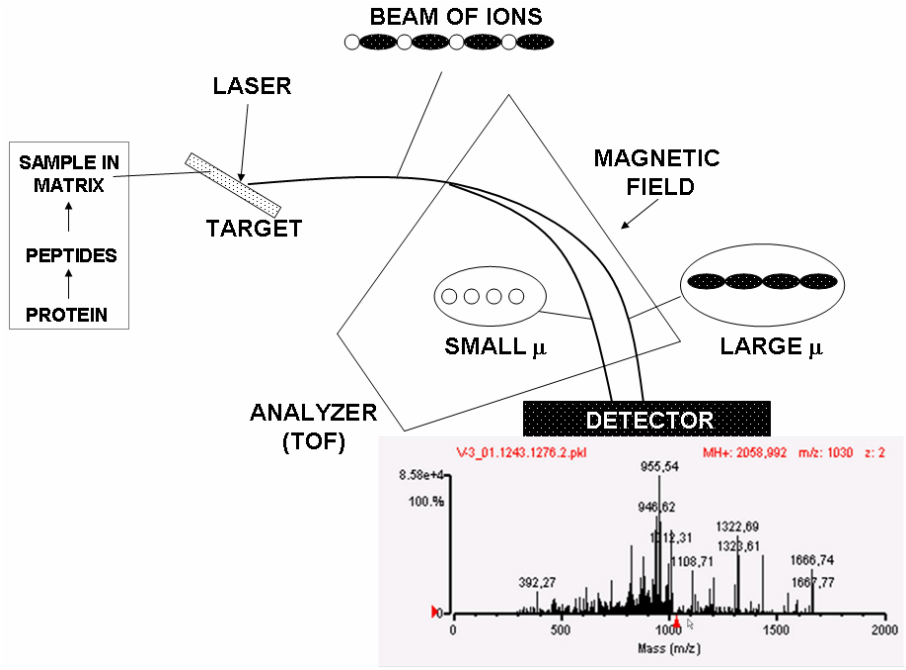

Fig. 3 Outline of MALDI-TOF. Sample is mixed with matrix (e.g., sinapinic acid) on a target. A laser beam impacts on this imparting sufficient energy to peptides or proteins to propel them through the TOF analyser. This estimates the time required for each peptide type to reach the detector which depends on momentum (μ). From this time of flight, an accurate *m/z* value can be calculated. Matching masses of tryptic peptides to masses predicted from sequence databases identifies proteins of origin by PMF.

rise to the original digest (Palagi *et al.*, 2006; Aebersold and Mann, 2006b). This dependence on sequence databases means that protein identification by this method in species such as bivalves can be problematic. In such cases, identification may instead need to be based on recognizing sequence homology rather than identity representing a major limitation in identifying target proteins in ecotoxicology (Dowling and Sheehan, 2006; McDonagh and Sheehan, 2007). High-resolution steps such as capillary liquid chromatography (LC) or capillary electrophoresis CE can precede Tandem MS giving "hyphenated" techniques; LC-tandem MS (Powell *et al.*, 2006) and CE-tandem MS (Hu *et al.*, 2005) facilitating extremely high resolution analysis of complex mixtures. In "shotgun sequencing", a tryptic digest of the entire proteome is separated using two dimensional LC (e.g., ion exchange followed by reversed phase) followed by sequencing in tandem MS (McDonald and Yates, 2002; Peng *et al.*, 2003) (Fig. 6). The very large amounts of MS data generated by such experiments raise important questions for experimental design and statistical analysis which have recently been reviewed (Domon and Aebersold 2006b; Nesvizhskii *et al.*, 2007).

Proteomic studies in bivalves

Environmental toxicology has recently begun to embrace proteomic technologies as a set of techniques suitable for understanding biological responses to environmental stress (Dowling and Sheehan, 2007; Monsinjon and Knigge, 2007; Nesatyy and Suter, 2007). As is usual with a new field, the approach has been at first tentative with an early emphasis, for example, on identifying PES rather than specific proteins. However, increasingly sophisticated proteomics technologies such as DIGE and SELDI-TOF have gradually been introduced in important landmark studies. This work has revealed a number of points of difficulty some of which are further explored near the end of this article. For example, when studying bivalves significant care must be taken when selecting the study organism with respect to size, sensitivity to stress, availability and previously published data. Moreover, tissues from different organisms may not necessarily be comparable for physiological reasons: mussels are filter feeders while clams are generally sediment feeders. Therefore, proteomic responses in gill from both species might be very different. Notwithstanding these practical limitations, proteomics is now increasingly accepted as a valid means of detecting often subtle effects of environmental pollutants. While not all of the following studies refer explicitly to oxidative stress, they often feature model compounds thought to function as pro-oxidants such as metals and hydrocarbons.

In an early application of 2DE to bivalves, Shepard *et al.* (2000) defined a PES for mussels exposed to copper or high salinity. Sets of proteins induced or repressed were identified as a sort of fingerprint for stress. Although this approach allowed

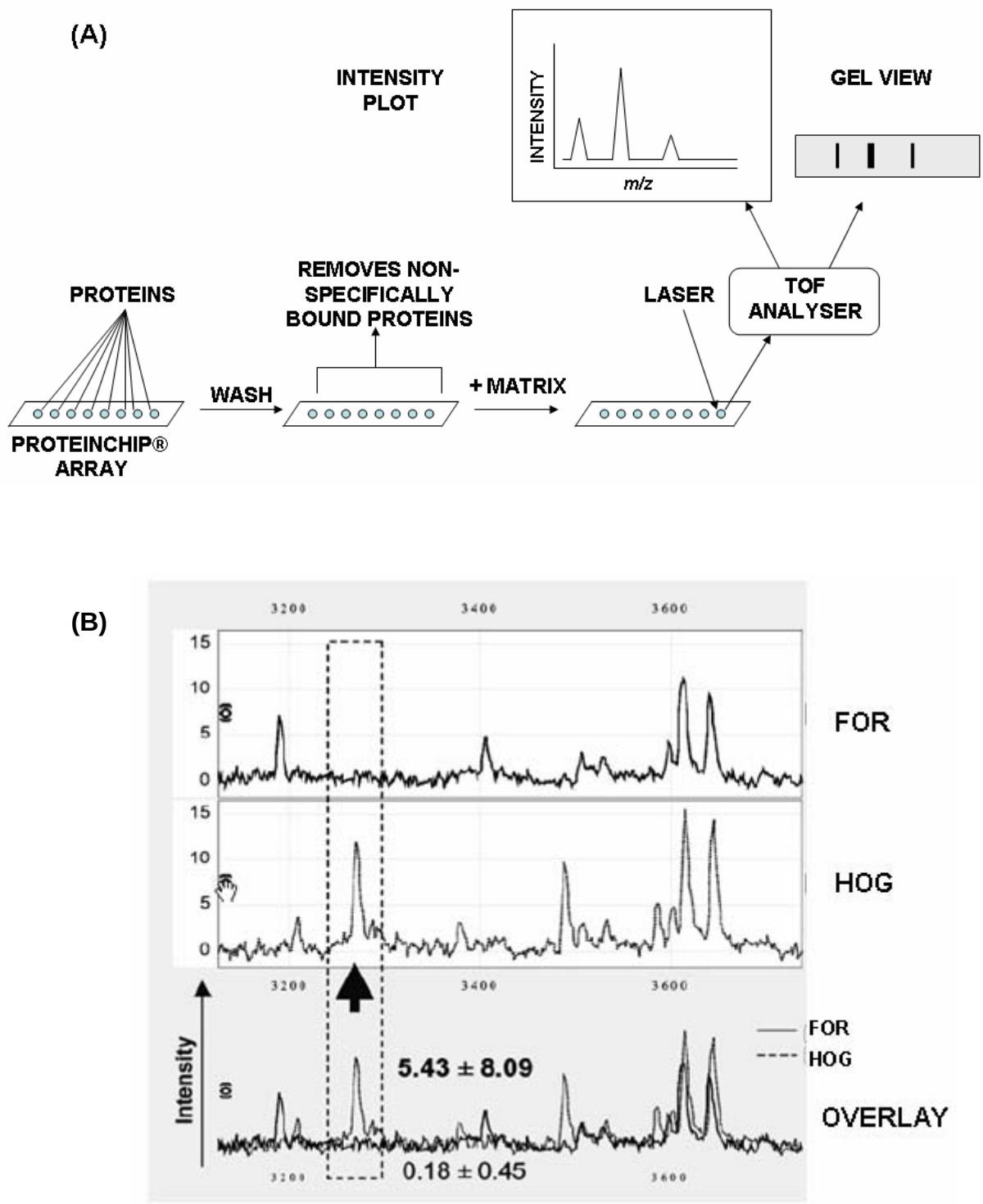

Fig. 4 (A) Outline of SELDI-TOF. Multiple protein samples can be added to ProteinChip® arrays which select proteins based on ion exchange, reversed phase or affinity interactions with groups immobilised on the array. Use of 8-well or 96-well arrays allows scaling to number of samples required with high throughput. Non-specifically-bound proteins are removed by washing and matrix (e.g., sinapinnic acid) is added. A laser beam propels proteins through a TOF analyser. The output may be represented as a plot of intensity versus *m/z* or as a synthetic "gel view" (i.e. how the sample might appear if separated by 1D SDS PAGE). **(B)** Selection of results of SELDI-TOF from Knigge *et al.* (2004) (reproduced with permission). A protein with *m/z* = 3268 was up-regulated in *M. edulis* digestive gland proteomes from animals in sites contaminated with PAH (HOG; n = 45) in comparison with reference animals (FOR; n = 39). From Knigge T, Monsinjon T, Andersen OK. Surface-enhanced laser desorption/ionization-time of flight-mass spectrometry approach to biomarker discovery in blue mussels(*Mytilus edulis*) exposed to polyaromatic hydrocarbons and heavy metals under field conditions. Proteomics 4: 2722-2727, 2004. Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission.

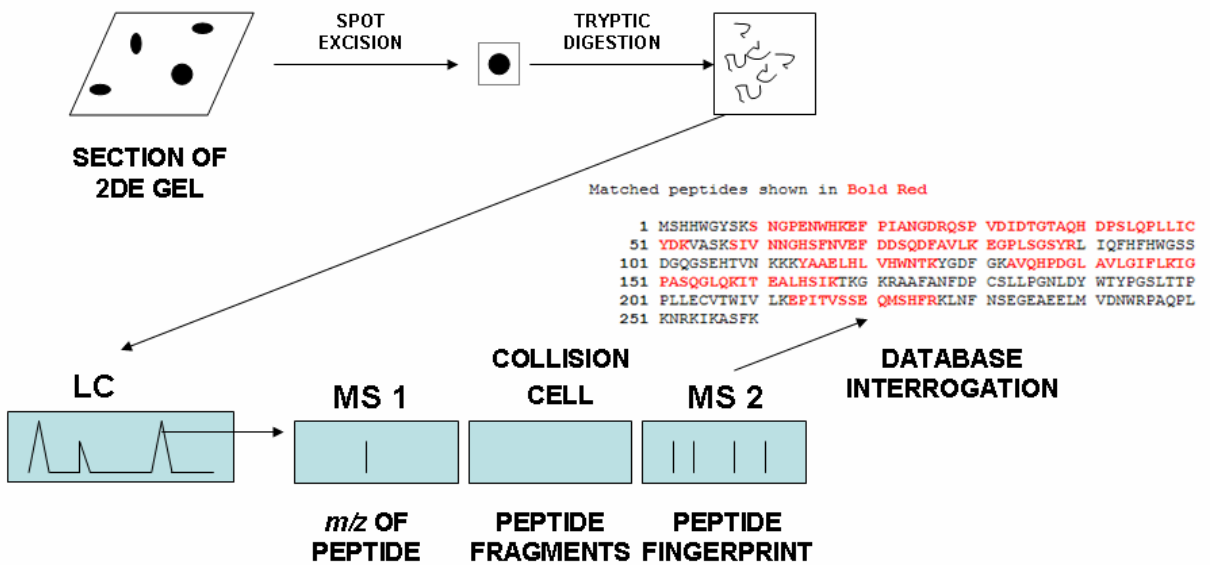

Fig. 5 LC-Tandem MS. Proteins in 2DE spots are digested with trypsin to form tryptic peptides. These are separated by capillary HPLC followed by *m/z* determination in first MS sector. Peptides are then fragmented in a collision cell and fragments are analysed in a second MS sector. Because proteins/peptides fragment in predictable ways, the series of fragments can be interpreted as a sequence. Sequences of several tryptic peptides are searched in sequence databases to give high-confidence identification.

distinction between two samples without any protein identification, it revealed little about underlying mechanisms of toxicity. The first bivalve study combining 2DE with MS identification was in the clam *Chamaelea gallina*, exposed to four model pollutants (Rodriguez-Ortega *et al.*, 2003). The 15 most dramatically altered protein spots were excised and analysed by MALDI-TOF MS, of which 4 proteins were identified. However, these proteins were cytoskeletal in origin which may point to high relative abundance, prevalence of cytoskeletal proteins in sequence databases or a cytoskeleton-related oxidative stress response (Mirabelli *et al.*, 1989). This approach was later extended to another clam species, *Scrobicularia plana*, sampled from sites of varying pollution status leading to identification of site-specific PES, identification of sequence tags for 16 proteins of increased abundance and unambiguous identification of more abundant hypoxanthine guanine phosphoribosyl transferase and glyceraldehyde 3-phosphate dehydrogenase (Romero-Ruiz *et al.*, 2006). As a further development Manduzio *et al.* (2004), identified 19 protein spots in gills of mussels exposed to crude oil. Again, some cytoskeletal proteins were identified but also some antioxidant and metabolic enzymes such as GST, heavy metal binding proteins and aldolase. The authors also reported that, although a number of spots produced good MALDI-TOF spectra, they did not yield a significant score for peptide mapping and hence identification. This highlights the difficulty in identifying bivalve proteins when they are so poorly represented in sequence databases, a recurring theme in bivalve proteomics (Dowling and Sheehan, 2007; Monsinjon and Knigge, 2007).

Knigge *et al.* (2004) applied a SELDI-TOF approach for biomarker discovery to mussels exposed to PAHs and heavy metals. SELDI-TOF should be capable of giving a proteomic profile of changes in cells under stress conditions as well as being well suited for low abundance and small proteins. However, a major problem encountered in this study was high variability amongst individual samples. These authors went on to combine protein array technology with SELDI-TOF of mussel serum from animals exposed to oil either on its own or spiked with alkylphenols (Bjørnstad *et al.*, 2006). A total of 83 mass peaks were perturbed in the spiked samples while only 49 were altered by oil alone. As with earlier studies, individual proteins were not explicitly identified but the mass patterns could be used as a fingerprint of effect. These studies demonstrate the power of SELDI-TOF for detection of effects when processing relatively large sets of individual complex protein samples although, again, analysis of such datasets is not without its difficulties (Monsinjon *et al.*, 2006).

DIGE technology was applied to digestive gland peroxisome-enriched fractions of *M. edulis* exposed under controlled conditions to model pollutants (Apraiz *et al.*, 2006; Mi *et al.*, 2007). These workers reported significant changes between groups and that PES identified could be used to assess oil exposure in marine pollution. A major advantage of this approach is that up- or down-regulated spots are readily identifiable on the same gel, thus improving identification by spot matching, reducing the number of gel replicates needed and raising the confidence levels for detection and quantification. DIGE also overcomes some of the shortcomings of traditionally stained gels which are susceptible to

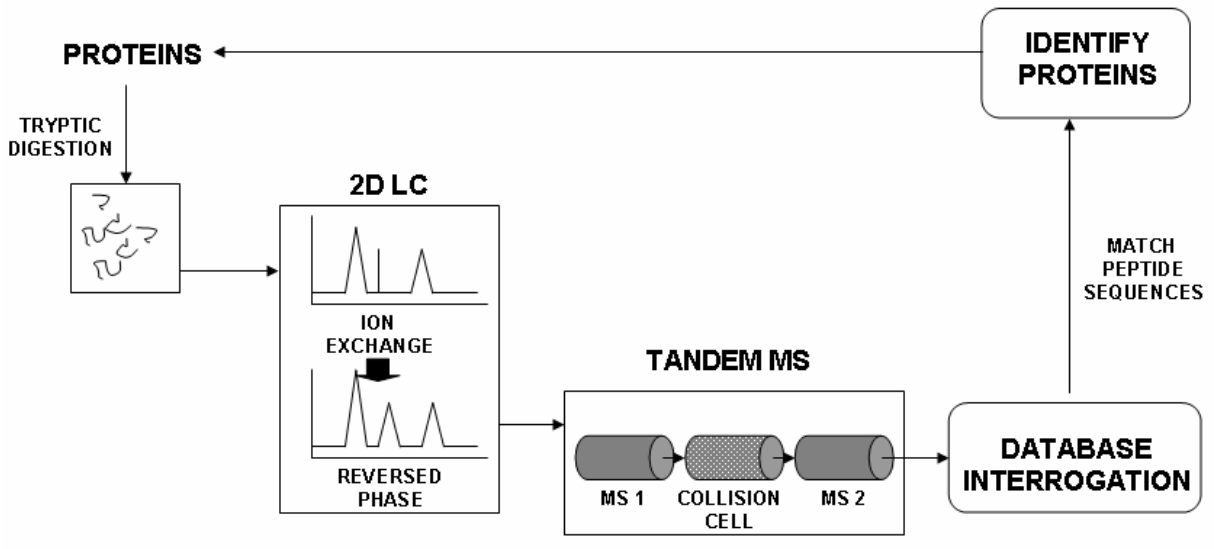

Fig. 6 Shotgun proteomics. This involves tryptic digestion of whole proteomes with high-resolution separation and identification technologies. In this schematic, tryptic peptides are first passed through 2D LC (ion exchange first with each peptide peak further separated by reversed phase) then each resultant 2D LC peak is passed to tandem MS. High-speed algorithms identify peptide sequences and thus allow identification of proteins in the starting proteome. Up to hundreds of proteins are identifiable in a single experiment.

variation in spot patterns, staining intensity and which have a low linear range (Nesatyy and Suter, 2007).

Oxidative stress can cause a range of reversible and irreversible modifications to proteins and their side chains. Some of these can lead to protein inactivation, some are protective of the protein's structural integrity and some may be viewed as sensing changes in the cell's redox status (Davies, 2005; Sheehan, 2006). Protein side chains can be irreversibly modified to aldehyde or ketone groups in a process termed carbonylation. Protein carbonylation can lead to protein aggregation, inactivation and degradation (Levine *et al.*, 1990; Davies, 2005). The 2DE proteomic approach can be combined with immunoblotting to investigate the carbonylation response in individual tissues in response to pollutants known to induce oxidative stress in both mussels and clams (McDonagh *et al.*, 2005; Dowling *et al.*, 2006; McDonagh and Sheehan, 2006). These studies revealed that levels of carbonylated proteins increased in general with oxidative stress although there was a tissue-specific response between organisms and the response was also pollutant-specific. Damaged proteins are removed from cells by proteolysis, mainly via the ubiquitin proteasome pathway (UPP) (Marques *et al.*, 2004). The UPP is responsible for selective digestion of many short lived intracellular regulatory proteins or abnormal cytosolic or nuclear proteins (Marques *et al.*, 2004). Changes in ubiquitination levels have been studied in bivalves by immunoblotting combined with 1DE in response to temperature and season (Hofmann and Somero, 1995; Buckley *et al.*, 2001) and in *M. trossulus* exposed to Exxon Valdez oil (Downs *et al.*, 2002). This approach has been extended to 2DE, for analysing mussels and clams exposed to a variety of oxidative stressors (McDonagh and Sheehan, 2006; Chora *et al.*, 2008; Tedesco *et al.*, 2008). Somewhat surprizingly, the ubiquitination PES is distinct from that for carbonylation suggesting that carbonylated proteins are degraded in a UPP-independent manner.

Irreversible modifications are most likely indices of protein damage, whereas reversible modifications such as glutathionylation and the formation of inter- or intra- disulphide bonds can play a regulatory role both in sub-stress scenarios (Biswas *et al.*, 2006; Ying *et al.*, 2007; Oktyabrsky and Smirnova, 2007; Poole and Nelson, 2008) and in the organism's response to oxidative stress (Stadtman and Levine, 2000). Gill tissues of *M. edulis* give a greater increase in glutathionylation in response to H_2O_2 treatment than digestive gland (McDonagh *et al.*, 2005). Gills of mussels exposed to H_2O_2 and menadione, as well as Baltic mussels sampled from a salinity gradient and exposed to pollutants were analysed using a diagonal electrophoresis technique to reveal formation of inter- and intrachain disulphides in proteins (McDonagh *et al.*, 2006; McDonagh and Sheehan, 2007, 2008; Prevodnik *et al.*, 2007). In this technique proteins are initially run in a non-denaturing primary dimension, the gel lane is then excised, proteins reduced in-gel and the lane is then placed orthogonally over a second dimension gel. Proteins not involved in inter-or intrachain disulphides fall along a diagonal while those involved in interchain disulphides will fall below the diagonal and those involved in intrachain disulphides will fall above the diagonal. It was found that actin and protein disulphide isomerase are involved in a number of disulfide bonds. As many proteins form disulphides in response to oxidative

stress, the method of Baty *et al.* (2005) was adapted to analyse changes in thiol status within the proteome. In this approach, free thiols are initially blocked with N-ethylmaleimide, disulphides reduced by DTT and resulting free thiols labelled with the thiol- reactive fluorescent compound, 5-iodoacetamidofluorescein (5-IAF). This enabled identification of a number of menadione-responsive proteins in the gills of *M. edulis* by LC-MS/MS (McDonagh and Sheehan, 2007, 2008). Another technique that uses an affinity column to pre-select for a subproteome in combination with 2DE has also been used in the clam *Tapes semidecussatus*, where a GSH-agarose column was used and tissue-specific expression of Pi class GST's was discovered (Dowling *et al.*, 2006). These studies take a sub-proteome approach to oxidative stress in bivalves considerably simplifying subsequent analysis.

Conclusions and perspectives

Recent years have seen development of new proteomic techniques, many of which have yet to be fully embraced by environmental toxicology. Some of these techniques such as "shotgun proteomics" necessitate fully-covered genome sequences which currently precludes many bivalves. Others, such as blue native gel electrophoresis have especial promise for studies of oxidative stress as this technique resolves protein-protein complexes in systems such as mitochondria (Krause, 2006). In the coming years, significantly more sequence data are expected to become available (McKillen *et al.*, 2005; Dupont *et al.*, 2007; Tanguy *et al.*, 2008). This is especially important as "gel-free" techniques overcome many of the well-known shortcomings of 2DE such as the need for detailed image analysis, misidentification due to co-migration of proteins, under/over-selection of some proteins due to their physical properties or position in the cell (e.g., membrane proteins, are not suitable for 2DE separations) (Görg *et al.*, 2004).

One of proteomic's ultimate goals is to unearth networks of interactions to understand a particular biological state as well as to identify and quantify involvement of individual proteins (Domon and Aebersold, 2006a). Relative quantification between protein samples is a crucial aspect of analysis and 2DE, when combined with image analysis, allows relative spot comparisons to be made between gels. However, numerous gel replicates and careful consideration of total amount of protein on gels is necessary. As the dynamic range of many protein stains is limited, relative quantification can be difficult, although the newer fluorescent stains and dyes have been a significant improvement (Nesatyy and Suter, 2008). Some of the newer approaches for quantification include, isobaric tags for relative and absolute quantification (iTRAQ) allowing relative quantification between tandem mass spectra and use of tags of varying mass that label N-terminus and side-chains of all peptides. Samples are then pooled and analysed by MS/MS. Isotope coded affinity tag (ICAT) reagents that are cysteine-specific offer options especially relevant to redox proteomics. Cysteines are one of the most rarely used amino acids but are often highly-conserved and involved in the regulation of important cellular processes. ICAT can provide information about the oxidative state of individual proteins (Leichert *et al.*, 2008).

Many proteomic studies of oxidative stress in bivalves have so far been performed under laboratory-controlled conditions, where bivalves are subjected to high levels of contaminants or stressors. This is understandable as proteomics is relatively new to environmental monitoring, and researchers often use artificially high exposures to see if there is measurable response. The next step is to develop proteomic techniques to the point that they can reveal differences in the field at environmentally-relevant pollutant concentrations.

A major issue to be considered in environmental proteomics studies so far has been the repeated identification of multifunctional proteins such as those of the cytoskeleton. An analysis of 15 environmental proteomics studies indicated that actin, tubulin or myosin were identified in almost 50 % of studies (Monsinjon and Knigge, 2007). Whether this is due to the high abundance of these proteins, that they are highly conserved across species or that it is a genuine stress-response remains to be elucidated. The presence of high abundance proteins could mask the oxidative stress response of low abundance proteins that are modified to greatest degree. When initially designing a proteomics experiment it should be kept in mind what the overall objectives of the study are, as with some careful initial planning the maximum information may be gleaned from results. A major bottleneck in proteomic studies is how to analyse the final data. If the question posed is to look for changes in a particular protein, a univariate statistical analysis such as the Student's t-Test may be sufficient, while a multivariate analysis is more appropriate for looking at changes in patterns of expression of proteins (Karp and Lilley, 2007). These issues should be kept in mind as they will affect the number of biological and analytical replicates required. The issue of whether to pool samples from a number of individuals in a proteomic study has been a subject of much debate and has been extensively reviewed elsewhere (Monsinjon and Knigge, 2007; Karp and Lilley, 2007). Briefly, pooling of samples may reduce biological variation and enhance statistical performance which, in a 2DE approach that is laborious and time-consuming, has obvious advantages. On the other hand, one aberrant sample could negatively impact on the quality of a particular pool and any phenotypic data is impossible to distinguish. Subpooling has been suggested as an alternative approach but again some considerations include that the pool should be large enough to reflect the average population and that subpooling creates a hierarchical structure within the data (Karp *et al.*, 2005). A 3-year European Research Project BEEP (Biological Effects of Environmental Pollution in Marine Ecosystems) published in a special edition of *Aquatic Toxicology* evaluated the use of biomarkers determined in marine organisms as a means of assessment of chemical contamination (Pampanin *et al.*, 2006). This report included a

number of proteomic studies including 2DE and SELDI-TOF, and highlighted the need for multivariate statistical methods for interpretation of complex datasets. In general, careful initial planning and outlining of overall objectives can save time over the course of the study. Together with the increasing availability of complete gene and protein databases we should see further developments in the field of environmental monitoring of bivalves as a response to oxidative stress (McKillen *et al.*, 2005; Dupont *et al.*, 2007).

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